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## EXCI-CEST: Exploiting pharmaceutical excipients as MRI-CEST contrast agents for tumor imaging

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# UNIVERSITÀ DEGLI STUDI DI TORINO

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23 **Title page**

24 *Title*

25 **EXCI-CEST: exploiting pharmaceutical excipients as MRI-CEST contrast agents for tumor**  
26 **imaging**

27

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43

## 44    **Abstract**

45    Chemical Exchange Saturation Transfer (CEST) approach is a novel tool within magnetic resonance  
46    imaging (MRI) that allows visualization of molecules possessing exchangeable protons with water.  
47    Many molecules, employed as excipients for the formulation of finished drug products, are  
48    endowed with hydroxyl, amine or amide protons, thus can be exploitable as MRI-CEST contrast  
49    agents. Their high safety profiles allow them to be injected at very high doses. Here we investigated  
50    the MRI-CEST properties of several excipients (ascorbic acid, sucrose, N-acetyl-D-glucosamine,  
51    meglumine and 2-pyrrolidone) and tested them as tumor-detecting agents in two different murine  
52    tumor models (breast and melanoma cancers). All the investigated molecules showed remarkable  
53    CEST contrast upon i.v. administration in the range 1-3 ppm according to the type of mobile proton  
54    groups. A marked increase of CEST contrast was observed in tumor regions up to 30 min post  
55    injection. The combination of marked tumor contrast enhancement and lack of toxicity make these  
56    molecules potential candidates for the diagnosis of tumors within the MRI-CEST approach.

57

58

## 59    **Keywords**

60    Excipients, MRI, CEST, tumor, imaging, chemical exchange saturation transfer

61

## 62    **Chemical Compounds studied in this article**

63    Ascorbic acid (PubChem CID: 54670067);

64    Meglumine (PubChem CID: 8567);

65    Sucrose (PubChem CID: 5988);

66    N-acetyl-D-glucosamine (PubChem CID: 439174);

67 2-Pyrrolidone (PubChem CID: 12025);

68

## 69 **Abbreviations**

70 MRI: Magnetic Resonance Imaging

71 CEST: Chemical Exchange Saturation Transfer

72 i.v.: intravenous

73

## 74 **1. Introduction**

75 Medicines could not be made without the use of pharmaceutical excipients that contribute notably  
76 to guarantee efficacy and safety of the final pharmaceutical product (Casas et al., 2015). Moreover,  
77 excipients perform multiple functions, besides completing the formulation volume, such as  
78 improving bioavailability, administration and acceptance of the treatment by the patient (Loftsson,  
79 2015; Narayan, 2011; Wening and Breitzkreutz, 2011). Another fundamental characteristic of  
80 excipients is their pharmacological and toxicological inactivity that allows them to be used at high  
81 doses (Abrantes et al., 2016). Several natural products, simple substances and mixtures are  
82 commonly used in formulating medicines, with chemical structures that vary from small molecules  
83 to polymers.

84 Interestingly, most, if not all of these molecules, possess exchangeable protons (hydroxyl, amine,  
85 amide groups) that can be potentially detected by chemical exchange saturation transfer (CEST)  
86 magnetic resonance imaging (MRI) (van Zijl and Yadav, 2011; Vinogradov et al., 2013). This  
87 technique enables the indirect visualization of molecules via magnetization transfer between  
88 exchangeable protons and bulk water protons. By applying a selective radiofrequency irradiation to  
89 the mobile protons, the induced saturation is transferred to the bulk water protons, thus inducing a  
90 reduction of the water signal (Liu et al., 2013). Several natural molecules and polymers (glucose,

glycogen, glycosaminoglycan, sialic acid, gelatin) have already been exploited as MRI-CEST contrast agents, since these molecules have precedence of use with human exposure (Chan et al., 2012; Jin et al., 2017; Liang et al., 2015; Ling et al., 2008; Shinar et al., 2014; van Zijl et al., 2007; Walker-Samuel et al., 2013). Also metabolites, drugs and polypeptides/proteins have been investigated to demonstrate their capability to generate contrast within this approach (Bar-Shir et al., 2015; Bar-Shir et al., 2014; Cai et al., 2015; Haris et al., 2012; Li et al., 2016; Liu et al., 2016; Longo et al., 2014a; McMahon et al., 2008; Zaiss et al., 2013). Moreover, several diamagnetic CEST agents have been proposed as exogenous probes for tumor imaging (Geraldès and Laurent, 2009). However, diamagnetic molecules require high doses to discriminate their contrast from direct water saturation and from endogenous magnetization transfer effects, due to the small chemical shift difference from water.

These considerations limit the effective use of exogenous molecules as CEST agents to those possessing low *in vivo* toxicity. According to these considerations, researchers firstly turned their attention to already clinically approved contrast agents, such as iodinated contrast media, exploiting their high safety profile and FDA approval (Aime et al., 2005b; Longo et al., 2011). Consequently, radiographic agents have been exploited for assessing tumor microenvironment properties, including perfusion (Anemone et al., 2017; Longo et al., 2016b), acidosis (Chen et al., 2015; Jones et al., 2015; Longo et al., 2016a; Longo et al., 2014b; Sun et al., 2014) and for assessing renal functionality (Longo et al., 2013; Longo et al., 2017).

Pharmaceutical excipients have attracted our interest since they can be used at very high dose due to their well-known safety profiles. In addition, excipients do not have any pharmacological effects, in contrast to active pharmaceutical ingredients. Ideally, a MRI-CEST contrast agent should possess good solubility and high safety profile, it should accumulate enough in the region of interest to produce contrast; afterwards, it should be excreted through the kidneys without long-term accumulation (Aime et al., 2005a; Sherry et al., 2009). The present investigation reports the MRI-

116 CEST properties of several pharmaceutical excipients (sucrose, N-acetyl-D-glucosamine, ascorbic  
117 acid, meglumine and 2-pyrrolidone), as novel, biocompatible MRI contrast agents for molecular  
118 imaging of tumors. We describe the *in vitro* MRI-CEST contrast enhancing properties and the *in*  
119 *vivo* investigation of these molecules in two murine tumor models.

120

## 121 **2. Methods**

### 122 *2.1 Materials*

123 All chemicals (Sucrose, N-acetyl-D-glucosamine, Meglumine, 2-pyrrolidone, Ascorbic acid) were  
124 purchased from Sigma-Aldrich (Sigma Aldrich, Milan, Italy).

125

### 126 *2.2 In vitro MRI CEST acquisition*

127 Phantoms containing vials of phosphate buffer solution of Sucrose, N-acetyl-D-glucosamine,  
128 Meglumine, 2-pyrrolidone or ascorbic acid were prepared at a concentration of 30mM and titrated  
129 over a range 6-7.4 pH units. CEST-MRI experiments were performed on a vertical 7 T MRI scanner  
130 Bruker Avance 300 (Bruker, Ettlingen, Germany) using a fast spin-echo sequence with centric  
131 encoding after presaturation pulses varying in power (1.5, 2.0, 3.0 and 6.0  $\mu$ T) for 5 or 7s at 37°C.  
132 A modified RARE sequence including a magnetization transfer module was used to acquire CEST-  
133 weighted images from -10 to +10 ppm with increments of 0.1 ppm around the water resonance.

134

### 135 *2.3 Cell lines for xenograft tumor models*

136 TS/A cells, derived from a metastasizing mouse cell line, originated from a mammary  
137 adenocarcinoma which arose spontaneously in a BALB/c female, were grown in RPMI 1640  
138 medium supplemented with 10% fetal bovine serum (FBS), 100U/mL Penicillin with 100  $\mu$ g/mL  
139 Streptomycin (Pen/Strep) and 2mM L-Glutamine (Nanni et al., 1983). B16-F10 cells, an

140 established murine melanoma cell line, were cultured in DMEM supplemented with 10% FBS, 100  
141  $\mu\text{g/ml}$  penicillin and 100  $\mu\text{g/ml}$  streptomycin. B16-F10 cells were obtained from American Type  
142 Culture Collection (ATCC).

143

#### 144 *2.4 Animal experiments*

145 6-old-week female BALB/c mice (n=5 for each molecule) were inoculated subcutaneously with 2.5  
146  $\times 10^5$  TS/A cells in 100  $\mu\text{L}$  of PBS on both flanks and 6-old-week male C57BL/6 mice (n=5 for  
147 each molecule) were inoculated subcutaneously with  $3 \times 10^5$  B16-f10 cell in 100  $\mu\text{L}$  of PBS on both  
148 flanks. BALB/c and C57BL/6 mice (Charles River Laboratories Italia S.r.l., Calco Italia) were  
149 maintained under specific pathogen free conditions in the animal facility of the Molecular  
150 Biotechnology Center, University of Turin, and treated in accordance with the EU guidelines  
151 (EU2010/63). All in vivo studies were conducted according to approved procedures of the  
152 Institutional Animal Care and Use Committee of the University of Torino.

153 Before imaging, mice were anaesthetized with a mixture of tiletamine/zolazepam (Zoletil 100;  
154 Vibac, Milan, Italy) 20mg/kg and xylazine (Rompun; Bayer, Milan, Italy) 5mg/kg and during the  
155 acquisition their breath rate was monitored throughout in vivo MRI experiments using a respiratory  
156 probe. Cannulation of the lateral tail vein with a catheter was exploited for intravenous injection of  
157 the investigated molecules.

158

#### 159 *2.5 In vivo MRI CEST acquisition and analysis*

160 A Bruker 7T Avance 300 MRI scanner (Bruker Biospin, Ettlingen, Germany) equipped with a 30  
161 mm  $^1\text{H}$  quadrature coil was used to scan mammary adenocarcinoma (TS/A cell line) and melanoma  
162 (B16-f10 cell line) tumor bearing mice 15 days post-inoculation. After the scout image acquisition,  
163  $T_{2w}$  anatomical images were acquired with a Fast Spin Echo sequence and the same geometry was



164 used for the following CEST experiments. CEST images were acquired with a single shot FSE  
165 sequence with centric encoding (TR: 6000 ms, TE: 4.0 ms) after a CW-RF presaturation pulse of  $B_1$   
166 = 1.5  $\mu$ T x 5s from a single axial slice with high in-plane resolution of 234  $\mu$ m (FOV 3 cm, MTX  
167 96, zero filled to 128, slice thickness 1.5mm) with 55 frequency offsets unevenly spaced in the  
168 range  $\pm 10$  ppm. Each investigated molecule was administrated intravenously at the dose of 1.2 g/kg  
169 b.w. with a single bolus of 100  $\mu$ L followed by continuous infusion at a rate of 500  $\mu$ L/h and CEST  
170 images were acquired before and every 10 minutes up to 30 minutes.

171 CEST images were analyzed using homemade scripts implemented in MATLAB (The Mathworks,  
172 Inc, Natick, MA). The Z-spectra were interpolated, on a voxel-by-voxel basis, by smoothing  
173 splines,  $B_0$ -shift corrected and saturation transfer efficiency (ST%) was measured by punctual  
174 analysis at 1.2 ppm (Terreno et al., 2009). For in vivo images, difference contrast maps ( $\Delta$ ST%)  
175 were calculated by subtracting the ST contrast after each molecules injection from the ST contrast  
176 before the injection on a per voxel basis. Extravasation fraction of each molecule was calculated as  
177 the percentage of pixels showing a  $\Delta$ ST% above the threshold (2%) in the manually-defined tumor  
178 region of interest.

179

## 180 *2.6 Statistical analysis*

181 Calculations were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA)  
182 software package; data are presented as mean  $\pm$  SD unless otherwise stated. Statistical significance  
183 was established at  $P < 0.05$ .

184

## 185 **3. Results**

### 186 *3.1 In vitro characterization of CEST properties*

187 The investigated molecules differ for the types of mobile protons, i.e. belonging to the hydroxyl,  
188 amide and amine groups, and for the number of exchanging protons (Figure 1). The ability to yield  
189 CEST contrast in the MR images is shown in Figure 2, where the contrast efficiency (ST effect) is  
190 plotted as a function of the chemical shift. Sucrose, ascorbic acid, meglumine and N-acetyl-D-  
191 glucosamine show CEST contrast peaking at ca. 0.7-1.2 ppm, due to the presence of hydroxyl  
192 protons. 2-Pyrrolidone showed a small CEST contrast at 0.7 ppm downfield from water, due to the  
193 presence of a cyclic amide (lactam) group. Both meglumine and N-acetyl-D-glucosamine showed,  
194 in addition to the less shifted hydroxyl protons, a second broad CEST contrast peak between 2 and  
195 3.5 ppm, due to amine and amide protons, respectively. As shown in Figure 2, when keeping  
196 constant the saturation field strength ( $1.5 \mu\text{T} \times 7\text{s}$ ), the CEST contrast showed a significant  
197 dependence with pH. The hydroxyl mobile protons of sucrose showed higher CEST contrast values  
198 at lower pH values (Figure 3A). Conversely, the hydroxyl protons of N-acetyl-D-glucosamine,  
199 meglumine and ascorbic acid showed a steady increase of the CEST contrast at high pH values, at  
200 all the investigated saturation field strengths ( $1.5 - 6 \mu\text{T}$ , Figures 3B, D and E). The CEST contrast  
201 of 2-pyrrolidone was almost independent from pH (Figure 3C). Sucrose, meglumine, ascorbic acid  
202 and N-acetyl-D-glucosamine showed in vitro a ST efficiency close to 1% per 1 mM concentration  
203 (irradiation pulse of  $1.5 \mu\text{T} \times 5\text{s}$ , pH 7,  $T=37^\circ\text{C}$ ; Figure 3F). The highest CEST contrast efficiency  
204 observed for sucrose (1.5 % ST per 1 mM concentration) likely depends on the large number of  
205 mobile protons (8  $-\text{OH}$  protons), whereas 2-pyrrolidone, having only one exchanging proton,  
206 showed the lowest CEST contrast.

207

### 208 3.2 *In vivo* CEST detection in tumor murine models

209 Two cancer xenograft models, TS/A (highly metastatic mouse breast cancer cells) and B16 (mouse  
210 melanoma cancer cells) were used for *in vivo* experiments. CEST agents were administered at the  
211 same dose by intravenous injection through the tail vein. A pronounced increase in the CEST

contrast in both TS/A and B16 tumor models for all the investigated molecules, with an average  $\Delta ST$  increase in the range 2- 6% in comparison to the pre-contrast ST values was observed (Figure 4). Sucrose showed a slightly higher CEST contrast in the B16 model ( $\Delta ST=4.1 \pm 0.7\%$ ) in respect to TS/A tumors ( $\Delta ST=2.7 \pm 0.5\%$ ) at all the investigated time points (Figure 4A). N-Acetyl-D-glucosamine slightly raised the CEST effect from the baseline with no difference between the two tumor models ( $\Delta ST=2.1 \pm 0.5\%$  and  $2.5 \pm 0.5\%$  for B16 and TS/A, respectively, Figure 4B). The CEST signal of meglumine increased by  $4.1 \pm 1.0\%$  for the B16 tumors and by  $2.5 \pm 0.4\%$  for the TS/A tumors (Figure 4C), at 10 min post injection ( $P<0.05$ ). Ascorbic acid showed the highest enhancement in TS/A tumors ( $\Delta ST=5.4 \pm 1.1\%$ ) in comparison to the B16 model ( $\Delta ST=2.7 \pm 0.7\%$ ), with statistically significant difference already 10 min after the i.v. administration ( $P<0.05$ , Figure 4D). For all the investigated molecules the CEST contrast measured in tumors persisted up to 30 min after the administration. Representative CEST contrast maps overlaid on anatomical images show the differential enhancement pattern among the investigated excipients within the investigated two murine tumor models (Figure 5). In particular,  $\Delta ST$  images showed a marked increase in CEST contrast in B16 and TS/A tumors for meglumine and ascorbic acid, respectively. The percentage of the tumor pixels where the CEST effect was detectable ( $\Delta ST$  higher than 2%) is dependent on both the molecules and the tumor model (Figure 6). In particular, CEST contrast increase was higher for the B16 model than for the TS/A one. Sucrose was detected in 40-60% of the tumor area according to the tumor model, whereas all the other molecules showed a detection fraction lower than 50% of the whole tumor volume.

232

#### 233 4. Discussion

234 This study demonstrated that several excipients can be exploited as MRI-CEST contrast agents for  
235 tumor detection in mice. A moderate to marked increase in CEST contrast in the tumor region was  
236 detected up to 30 min following intravenous administration, according to the exploited agents or the

237 investigated tumor models. Meglumine and ascorbic acid yielded the highest contrast enhancement  
238 ( $\Delta ST > 5\%$ ) in B16 and TS/A models, respectively (Figure 4). Conversely, lower CEST  
239 enhancements were measured for sucrose and N-acetyl-D-glucosamine. The potential of glucose  
240 and its derivatives to act as MRI-CEST agents for tumor imaging has already been demonstrated  
241 (Chan et al., 2012; Walker-Samuel et al., 2013; Xu et al., 2015). However, for glucose, the main  
242 drawback was associated to its rapid metabolism once entered in the tumor cells, with consequent  
243 reduction of CEST contrast capabilities. For this reason, glucose analogs, such as 2-deoxy-glucose  
244 (2DG) and 3-oxy-methyl-glucose (3OMG) have been proposed as they showed superior contrast  
245 efficiency owing to the reduced metabolic conversion in the case of 2DG (Nasrallah et al., 2013;  
246 Rivlin et al., 2013) or to the lack of metabolic transformation in the case of 3OMG (Rivlin et al.,  
247 2014). As a consequence, such derivatives provide an improved and long-lasting CEST contrast in  
248 mice carrying xenograft tumors. On the other hand, the safety of these compounds has still to be  
249 demonstrated, since the high concentrations ( $> 1\text{--}1.5\text{ g/kg b.w.}$ ) required to generate enough CEST  
250 contrast might limit their use to laboratory animals. An analogous limitation may be envisaged for  
251 the recently reported CEST agents based on salicylic acid (a metabolite of aspirin), although their  
252 very large chemical shift difference (unusual for diamagnetic CEST agents) holds promise for  
253 applications at lower magnetic fields (Lesniak et al., 2016; Yang et al., 2013). Conversely, the  
254 herein investigated excipients hold a very high safety profile, considering that they are used at high  
255 dosages to provide suitable formulations for drugs.

256 In contrast to N-acetyl-D-glucosamine and ascorbic acid that can be metabolized within the body,  
257 sucrose (when injected i.v.) and meglumine are rapidly excreted unchanged in the urine, with no  
258 evidence for metabolism (Heeg et al., 1977). Clearly, this represents a great advantage in  
259 comparison to metabolizable probes that do not accumulated in the extracellular extravascular space  
260 of tumors. Furthermore, metabolic products cannot provide enough CEST contrast as the original  
261 molecule, with a following decrease of their observed CEST contribution. This may partly explain

262 the non-optimal CEST contrast in tumors upon N-acetyl-D-glucosamine administration, in  
263 comparison to the other excipients, despite the high *in vitro* CEST contrast efficiency. Similar  
264 findings for N-acetyl-D-glucosamine were observed by Navon group, who investigated the CEST  
265 properties of this molecule and of glucosamine as alternatives to glucose analogs (Rivlin and  
266 Navon, 2016). In our study, the observed increase in CEST contrast in tumors with N-Acetyl-D-  
267 Glucosamine was lower ( $\Delta ST = 2\text{-}3\%$ ) than what previously reported ( $\Delta ST = 6\text{-}7\%$ ), albeit a  
268 similar dosage was administered. These observations may be accounted by the use of different  
269 tumor cell lines or by the lower saturation pulse power that has been applied in our experimental  
270 protocol ( $1.5\ \mu\text{T}$  vs  $2.4\ \mu\text{T}$ ).

271 Meglumine showed distinct contrast enhancement capabilities between TS/A and B16 tumors.  
272 Meglumine is not internalized inside cells, therefore differences in CEST contrast enhancements are  
273 only dependent on the accumulation within the extracellular extravascular space, hence reflecting  
274 different vascularization properties. As such, this molecule can be considered an extracellular-fluid  
275 agent analogous to the clinically approved Gadolinium-based contrast agents (Morana et al., 2013)  
276 or to Iodine-containing X-ray systems (Rutten and Prokop, 2007). Thus, meglumine may be an  
277 attractive candidate to be used as MRI-CEST contrast agents for tumor imaging with remarkable  
278 contrast efficiency.

279

## 280 5. Conclusions

281 The herein investigated excipients show remarkable MRI-CEST properties as demonstrated by the  
282 *in vivo* visualization of tumors in two murine models. The extremely good safety profile of the  
283 excipients provides support to the view that these systems may be considered reliable candidates for  
284 clinical translation.

285

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288

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## 419 **Figure Legends**

420 **Figure 1.** Chemical structures and molecular weights (g/mol) of the investigated excipients.

421 **Figure 2.** CEST contrast curves for the investigated molecules (sucrose, black; N-acetyl-D-  
422 glucosamine, red; 2-Pyrrolidone, grey; meglumine, green; ascorbic acid, blue) obtained at  
423 concentration of 30 mM at pH 6 (A) and pH 7.4 (B) using a saturation power level of 1.5  $\mu$ T with  
424 duration of 7s at 7T and 37°C.

425 **Figure 3.** CEST contrast dependence on pH measured in the range of 6-7.4 pH units at different  $B_1$   
426 levels (saturation power from 1.5  $\mu$ T to 6  $\mu$ T for 5s, 7T, 37°C) for sucrose (A), N-acetyl D-  
427 glucosamine (B), 2-Pyrrolidone (C), meglumine (D) and ascorbic acid (E).

428 **Figure 4.** Box-plots showing averaged mean  $\Delta$ ST increase (calculated as ST post -ST pre injection)  
429 in TS/A (grey bars) and B16 (black bar) tumor regions using  $B_1 = 1.5\mu$ Tx5s on a 7T MRI scanner.  
430 CEST contrast observed after i.v. administration of sucrose (A), N-acetyl-D-glucosamine (B),  
431 meglumine (C) and ascorbic acid (D) at a dose of 1.2 g/kg b.w. was observed at 10, 20 and 30  
432 minutes post-injection.

433 **Figure 5.** Representative  $\Delta$ ST tumor maps superimposed on anatomical images showing CEST  
434 contrast increase for sucrose, N-acetyl-D-glucosamine, meglumine and ascorbic acid 20 min after  
435 i.v. administration using  $B_1 = 1.5 \mu$ T x 5s in B16 (top row) and TS/A (bottom row) tumors.

436 **Figure 6.** Box-plot showing extravasation fraction calculated as percentage of pixels with  $\Delta ST$   
437 higher than 2% for sucrose, N-acetyl-D-glucosamine, meglumine and ascorbic acid 20 min after i.v  
438 administration using  $B_1 = 1.5 \mu T \times 5s$  for B16 (A) and TS/A (B) murine tumors.